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PRINCIPAL INVESTIGATOR: Mark Onaitis, M.D.

CONTRACTING ORGANIZATION: Duke University
Durham, NC 27705

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Mark Onaitis, M.D.

E-Mail: mwo@duke.edu

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14. ABSTRACT

We continue to make progress on the proposed work. For the first aim, we have rederived and crossed a floxed p63 mouse line with our Scgb1a1-CreER; lsl Sox2-IRES GFP mouse line. The resulting mouse line has no decrement in carcinogenesis. We are currently deleting p63 in human lung cancer cell lines to further validate the finding that p63 is dispensible for Sox2-induced tumorigenesis. A manuscript describing this work is in preparation. In aim 2, we have attempted further transplants of proximal Sox2-expressing bronchial epithelial cells into the alveoli. However, we have not seen tumor formation even in the presence of supporting fibroblast cells. We are trying different adaptations to make this work, but it may be that proximal cells do not grow well in the distal environment when expressing Sox2. For aim 3, speed congenics continues to create pure strains of Scgb1a1-CreER; lsl Sox2-IRES GFP mice. Comparison of the mice will allow identification of modifier genes contributing to Sox2-induced lung cancer.

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Final Progress Report: The Mechanism of Sox2-Induced Lung Cancer

Introduction

The purpose of the proposed research is to clarify the mechanism of Sox2-induced non-small cell lung cancer. The first aim involves investigating p63 as a direct target of Sox2 in tumor cells. The second aim investigates the tumor-initiating ability of proximal and distal respiratory epithelial cells when Sox2 is overexpressed. The third aim tests the hypothesis that strain-specific modifier genes affect the phenotype of mice inducibly overexpressing Sox2 in Scgb1a1-expressing cells. We have continued work on all three aims.

Aim 1

We have made continued progress on this aim over the past year. The main purpose of the aim is to identify the importance of the p63 gene in Sox2-induced oncogenesis. Because p63 has been demonstrated to be important in the maintenance of squamous epithelia, because it leads to squamous metaplasia when overexpressed in the mouse, and because p63 is expressed in SOX2+ human squamous lung cancers and in SOX2+ basal cells, we hypothesized that p63 is a direct target of Sox2 and that it is essential in Sox2Õs ability to intiaite lung cancer. Because p63 is expressed in 2 different isoforms, we first developed primers for each isoform. Reverse transcription-polymerase chain reaction (RT-PCR) was carried out using these primers, and the results are depicted in Figure 1. As the figure demonstrates, the TA-p63 isoform is upregulated by Sox2 in our Scgb1a1-CreER-induced mouse model. The delta-N isoform is not detected.

The next goal was to perform chromatin immunoprecipitation (ChIP) using an antibody for Sox2. However, the small number of cells recovered per mouse (using digestion of the intraparenchymal lung epithelium followed by trypsinization and sorting for GFP) made performance of ChIP from these cells impractical. We thus changed course to use the SOX2-overexpressing human squamous cell lung carcinoma cell line H520. Expansion of these cells gave us enough material for efficient ChIP. After the cells were fixed and sonicated to shear the DNA, immunoprecipitation was performed using the Seven Hills Biochemical Sox2 antibody. Crosslinking was then reversed, and massively parallel sequencing was carried out using the Duke Sequencing core. Analysis of these sequence reads surprisingly demonstrated that p63 is not a direct target of SOX2. Thus, it may be that p63 is being upregulated by another direct target of SOX2. Of note, this analysis provided several other candidate gene targets of SOX2 that we will be characterizing over the next years.

The next item in the Statement of Work involves breeding an inducible p63-knockout allele into the Scgb1a1-CreER; Rosa26-Sox2-IRES GFP mouse line to assess the necessity of p63 for Sox2 induced tumorigenesis. Our close collaborator Barry Stripp provided re-derived inducible p63-knockout (floxed p63) mouse line from frozen embryos. We set up breeding cages to produce Scgb1a1-CreER; Rosa26-Sox2-IRES GFP; floxed p63 mice. Again surprisingly, deletion of p63 has no discernible effect on Sox2-induced oncogenesis in this model (Figure). Larger numbers of mice (43 in each group) over the past year have confirmed that there is no difference in phenotype between mice over expressing Sox2 and this over expressing Sox2 while p63 is simultaneously deleted. This required a great deal of sectioning and counting.

The last piece in a potential manuscript describing a non-essential role for p63 in SOX2-induced neoplasia is demonstration of minimal phenotype to knockdown of P63 in human squamous cell lung cancer cell lines. We have obtained constructs to make lentiviral shRNAs to knockdown p63 in the SOX2-hi H520 human squamous cell lung cancer line. The sequences are as follows.

CCGGCATCTGACCTGGCATCTAATTCTCGAGAATTAGATGCCAGGTCAGATGTTTTTTG

CCGGAGTTGCACTTATTGACCATTTCTCGAGAAATGGTCAATAAGTGCAACTTTTTTTG

We have transduced H520 cells with lentivirus encoding these shRNAs and are currently selecting the infected cells with antibiotics. These cells will then be used for *in vitro* proliferation assays and *in vivo* xenograft assays.

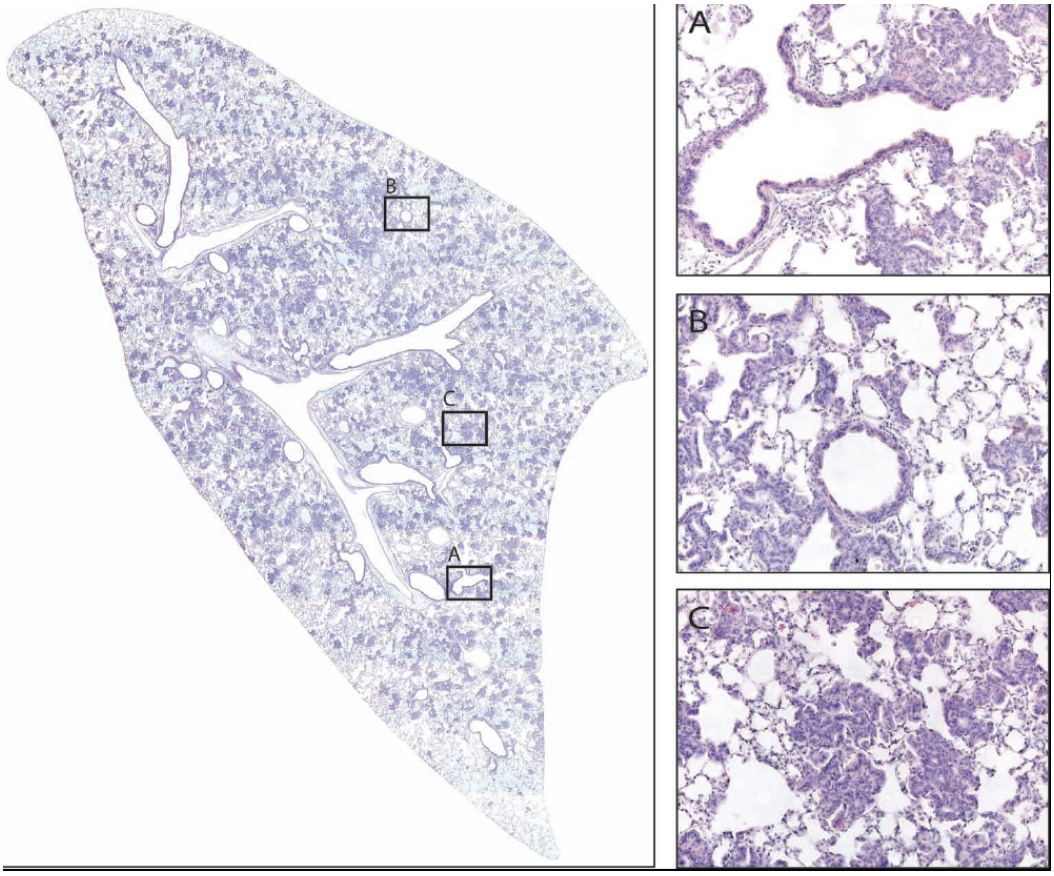

Figure 1: Representative image of a lung from Scgb1a1-CreER; lsl Sox2 IRES GFP; floxed p63 mouse. H&E stain.

Aim 2

The main goal of Aim 2 is to explore whether there is a microenvironmental contribution to Sox2-induced oncogenesis in the mouse. We planned to isolate proximal Scgb1a1-CreER; lsl Sox2 IRES GFP cells via flow sorting for transplantation into the retroorbital veins of immunodeficient Rag1-/- mice. While our preliminary data demonstrated that this transplantation led to tumors when the transplant was performed using K-RasG12D-expressing cells, we have not obtained tumors using the Sox2-overexpressing proximal cells. Possible explanations for this lack of tumor formation include lack of ability of the proximal cells to form tumors no matter the environment as well as a possible need for cotransplantation with stromal cells. However, transplants of distal epithelial cells have also not grown robustly. We have reasoned that the transplanted cells may require fibroblasts for support. We thus initiated transplants using mouse fibroblasts 1:1 with Sox2-overexpressing proximal and distal Clara cells. Again, no robust tumor formation was identified in the recipient mouse lungs.

Over the past year, we have tried several different adaptations to the protocol including using more cells, using different media when isolating the cells. However, in each case, we have failed to identify tumor formation on the transplanted lungs. We are currently working on a protocol to directly inject cells into the lung parenchyma in order to avoid the bloodstream. We will test this over the next year.

Aim 3

The final aim of the grant is to explore the role of modifier genes in Sox2-induced neoplasia. Approximately half of the Scgb1a1-CreER; Rosa26-Sox2-IRES GFP mice develop cancer on a mixed 129/C57/Bl6 background. We propose to breed our mixed mouse line to pure C57/Bl6, 129, and AJ backgrounds. The crosses have begun in our animal facility. Duke has initiated an in-house speed congenics program. The first progeny mice have been submitted for massively-parallel sequencing. This will direct further breeding.

We have continued in-house speed congeners over the past year but have not reached pure backgrounds yet. This goal was probably unrealistic for the short time frame of the grant. However, we will continue this project with other funds so that we can identify genetic modifiers impacting upon tumor formation when Sox2 is over expressed.

KEY RESEARCH ACCOMPLISHMENTS

- Determination of the isoform of p63 induced by Sox2 over expression
- Identification of direct SOX2 transcriptional targets by ChIP-sequencing
- Elucidation that p63 is dispensable for SOX2-induced tumorigenesis in the mouse alveoli

REPORTABLE OUTCOMES

As yet, we have no reportable outcomes. We have begun to put together a manuscript regarding the dispensability of p63 in Sox2-induced lung cancer. We envision at least 2 other manuscripts arising from the ChiP-sequencing. One of these will be on the role of Connective Tissue Growth Factor (CTGF) downstream of SOX2.

CONCLUSION

We believe that we are making steady progress toward understanding the mechanism of SOX2-induced tumoirgenesis. We should be able to complete manuscripts in the next 6 months.